

## Elimination of both E1 and E2a from Adenovirus Vectors Further Improves Prospects for In Vivo Human Gene Therapy

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**A novel recombinant adenovirus vector, Av3nBg, was constructed with deletions in adenovirus E1, E2a, and E3 regions and expressing a  $\beta$ -galactosidase reporter gene. Av3nBg can be propagated at a high titer in a corresponding A549-derived cell line, AE1-2a, which contains the adenovirus E1 and E2a region genes inducibly expressed from separate glucocorticoid-responsive promoters. Av3nBg demonstrated gene transfer and expression comparable to that of Av1nBg, a first-generation adenovirus vector with deletions in E1 and E3. Several lines of evidence suggest that this vector is significantly more attenuated than E1 and E3 deletion vectors. Metabolic DNA labeling studies showed no detectable de novo vector DNA synthesis or accumulation, and metabolic protein labeling demonstrated no detectable de novo hexon protein synthesis for Av3nBg in naive A549 cells even at a multiplicity of infection of up to 3,000 PFU per cell. Additionally, naive A549 cells infected by Av3nBg did not accumulate infectious virions. In contrast, both Av1nBg and Av2Lu vectors showed DNA replication and hexon protein synthesis at multiplicities of infection of 500 PFU per cell. Av2Lu has a deletion in E1 and also carries a temperature-sensitive mutation in E2a. Thus, molecular characterization has demonstrated that the Av3nBg vector is improved with respect to the potential for vector DNA replication and hexon protein expression compared with both first-generation (Av1nBg) and second-generation (Av2Lu) adenoviral vectors. These observations may have important implications for potential use of adenovirus vectors in human gene therapy.**

Recombinant adenovirus vectors are promising candidates for a number of potential human gene therapy applications (25, 26). The acceptability of these vectors for safe use in vivo in humans depends on both a favorable toxicity profile and a lack of autonomous vector replication. The knowledge that nearly all adenovirus functions, including viral replication, critically depend on expression of the adenovirus first early region (E1) (8, 11, 22) led to the construction of E1 deletion vectors for gene transfer (reviewed in reference 1). These replication-deficient, first-generation adenovirus (Av1) vectors were first used for in vivo gene transfer in animal models in the cotton rat lung (20, 21). Theoretically, Av1 vectors had several advantages in this model: (i) a natural tropism for the lung, (ii) the ability to infect dividing and nondividing cells, (iii) high efficiency in vivo gene transfer and expression, (iv) high titer ( $\sim 10^{11}$  infectious units per ml), and (v) relative replication deficiency (5, 11, 20, 21). Subsequently, Av1 vectors were shown to be efficient for in vivo transfer and expression of genes in a variety of tissues and organs in various animal models (reviewed in reference 26). However, studies with E1 deletion vectors from a number of laboratories have now demonstrated that expression of the transferred gene (transgene) is transient and accompanied by a significant host response to the vector (24, 26, 27, 31). This host response consists of initial nonspecific inflammation followed by specific cellular and humoral immune responses directed at adenoviral vector proteins (24, 26–28, 31). The relationship of inflammatory and humoral antibody responses to the duration of transgene ex-

pression is currently unclear. However, the relationship of the cytotoxic T-lymphocyte (CTL) response to adenovirus gene expression has led to the hypothesis that further attenuation of viral gene expression may improve the in vivo utility of these vectors by increasing the duration of transgene expression and by reducing host responses.

One attempt to create a more attenuated adenovirus vector (3) combined a temperature-sensitive (*ts*) mutation within the adenovirus DNA-binding protein (DBP) (4) together with the E1 deletion. The *ts* mutant DBP molecule is nonfunctional at higher temperatures (40.5°C) but functional at lower temperatures ( $\leq 37^\circ\text{C}$ ) (4). The design of this second-generation adenovirus (Av2) vector was based on the knowledge that the DBP, a product of the E2a gene, plays a role in molecular control of vector DNA replication and the tightly linked events of major late region gene expression (5, 9, 18). This Av2 vector showed a prolonged transient duration of transgene expression in mice compared with an Av1 vector and was associated with a reduced, but not absent, CTL response (3, 29).

In the present article we describe the construction of a novel vector with deletions in E1, E2a, and E3 regions which expresses a  $\beta$ -galactosidase reporter and demonstrate that this vector effects gene transfer comparable to that of an Av1nBg vector. Comparative molecular characterization of Av1nBg, Av2Lu, and Av3nBg vectors has been carried out in vitro in a variety of cell lines which specifically complement none, one, or both of the functionally relevant adenoviral E1 or E2a region deletions in these vectors. These studies have evaluated the potential for vector DNA replication, attenuated adenovirus gene expression from the vector backbone, and the efficiency of transgene transfer and expression. In the Av3nBg vector (and also in the previously constructed Av1nBg), the E3

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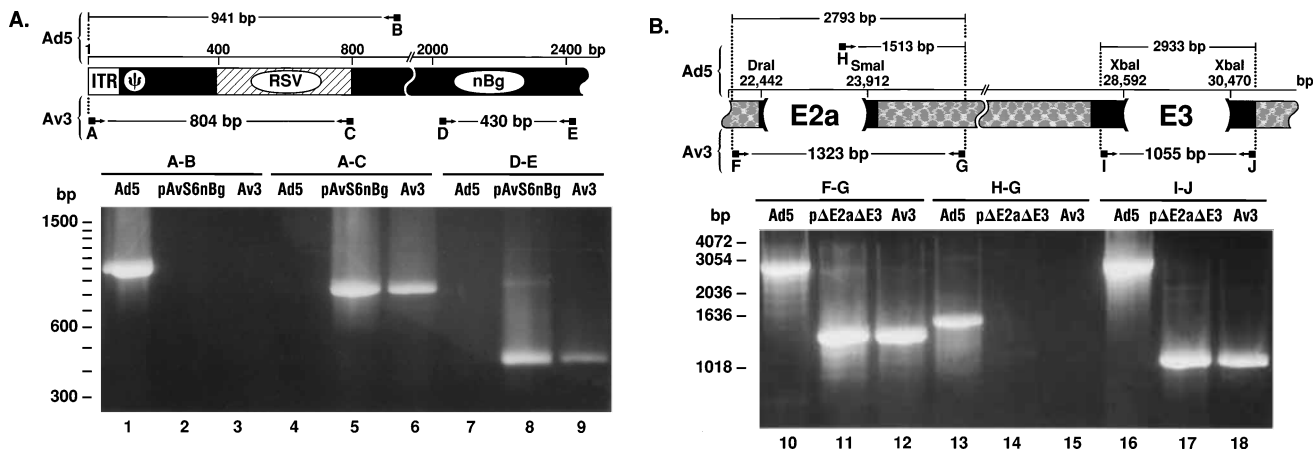


FIG. 1. Structural characterization of the Av3nBg DNA genome using PCR analysis. (A) Evaluation of the heterologous transgene expression cassette region. Av3nBg DNA is represented schematically, showing the locations of the left-end adenovirus inverted terminal repeat (ITR), encapsidation signal ( $\psi$ ), and the heterologous Rous sarcoma virus promoter (RSV) which drives expression from the heterologous nucleus-localizing  $\beta$ -galactosidase reporter transgene (nBg). The locations of PCR primers specific only to Ad5 sequences are shown above, while those of primers corresponding specifically to Av3nBg or both Ad5 and Av3nBg sequences are shown below. The direction of primer-directed PCR DNA synthesis is shown (arrows). The sizes of the expected PCR amplification products are indicated for the corresponding primer pairs. Certain restriction endonuclease cleavage sites used in construction are indicated above the vector along with their nucleotide positions. A photograph of agarose gel electrophoretic separation of PCR products is shown below. The sizes of marker DNA fragments (100-bp DNA ladder; Gibco-BRL) are indicated. The specific primer pairs used for each PCR amplification are shown above the gel along with the particular source of DNA for each amplification reaction. Ad5, wild type; pAvS6nBg, adenovirus construction plasmid used for construction of both the Av1 vector and the Av3 vector; Av3, Av3nBg vector. (B) Analysis of the vector genome in the E2a and E3 deletion regions. Molecular weight marker DNA (1-kb DNA ladder; Gibco-BRL) sizes are shown. The specific primer pairs used are indicated along with the DNA source. p $\Delta$ E2a $\Delta$ E3, plasmid used in construction of Ad5 $\Delta$ E2a $\Delta$ E3 and ultimately Av3nBg.

region was deleted because E3 region genes are not expressed in the absence of induction from E1a proteins and are not required for replication of these vectors (8, 11, 20).

**Construction of the Av3nBg vector.** The E1, E2a, and E3 deletion recombinant adenovirus vector, Av3nBg, expressing a nuclear targeted  $\beta$ -galactosidase reporter was constructed essentially as previously described (16) by homologous recombination between pAvS6nBg (25, 31) and the large *Cla*I fragment constituting the right side of a novel human adenovirus serotype 5 (Ad5) mutant. This mutant adenovirus, designated Ad5 $\Delta$ E2a $\Delta$ E3, contained deletions in adenovirus E2a and E3 regions. Ad5 $\Delta$ E2a $\Delta$ E3 was constructed in several steps beginning with the subcloning of the 3' end of the Ad5 genome (bp 21562 to 35936; nucleotide sequence coordinates refer to Ad5 [GenBank accession no. M73260] unless otherwise noted) as two separate restriction fragments in pBR322 for the creation of the E2a and E3 region deletions, respectively. For creation of the E2a deletion (Fig. 1), a *Dra*I-*Sma*I fragment (bp 22442 to 23912) was removed from a plasmid containing the Ad5 sequences spanning from *Bam*HI (bp 21562) to *Spe*I (bp 27080). The E3 deletion (Fig. 1) was created separately by removing an *Xba*I fragment (bp 28592 to 30470) from a plasmid containing the Ad5 sequences spanning from *Spe*I (bp 27080) to the 3' end of Ad5 (bp 35936). This resulted in an E3 region deletion identical to the one carried by the adenovirus mutant *Addl*-327 (23) and other adenoviral vectors (25, 26). A *Sal*II site was introduced into the latter E3 deletion plasmid 3' of the Ad5 sequences for cloning purposes. The Ad5 sequences from these respective E2a deletion and E3 deletion plasmids were then joined at the corresponding *Spe*I site (bp 27080) to create the plasmid p $\Delta$ E2a $\Delta$ E3 containing deletions in E2a (bp 22442 to 23912) and E3 (bp 28592 to 30470). The resultant plasmid was cleaved with *Bam*HI and *Sal*II, and the adenovirus fragment was ligated to the left-hand fragment of *Bam*HI-cleaved Ad5 DNA (bp 1 to 21562). This ligation mixture was then introduced into dexamethasone-induced KE2a cells by electroporation. KE2a is a stable cell line, derived from KB cells (ATCC

CCL-17), which expresses the Ad5 E2a gene (bp 24730 to 21562) under positive regulation of the glucocorticoid-responsive mouse mammary tumor virus promoter (pMAM Neo; Clontech, Palo Alto, Calif.). A similar E2a expression construct has been previously described (2). Transfected cells were lysed after 7 days by three cycles of freezing and thawing, and the lysate was used to infect fresh monolayers of KE2a cells. After 7 to 10 days, plaques representing Ad5 $\Delta$ E2a $\Delta$ E3 were recovered, and virus was purified. Plaque purification of Ad5 $\Delta$ E2a $\Delta$ E3 was then repeated, and stocks of Ad5 $\Delta$ E2a $\Delta$ E3 were prepared.

The Av3nBg vector was created after mixing pAvS6nBg DNA with the large *Cla*I DNA fragment of the Ad5 $\Delta$ E2a $\Delta$ E3 genome by cotransfection using Lipofectamine (Gibco-BRL, Gaithersburg, Md.) into dexamethasone (0.3  $\mu$ M)-induced AE1-2a cells to allow homologous recombination and subsequent vector replication. The AE1-2a cell line, derived from A549 cells (ATCC CCL-185), expresses the Ad5 E1 (E1a and E1b; bp 552 to 4090) genes under positive regulation of a minimal glucocorticoid response element (GRE5) promoter (U.S. Biochemicals, Cleveland, Ohio) and the E2a gene as described above for the KE2a cells. A detailed description of the AE1-2a cell line and its properties and capacity for production of Av1 and Av3 vectors will be given elsewhere. Crude viral lysate of transfected cells was prepared after 7 days and used to infect dexamethasone-induced AE1-2a cells. Twenty-four hours after infection, cells containing replicating,  $\beta$ -galactosidase-expressing vector were enriched by fluorescence-activated cell sorting (17). In this experiment, 10,000 fluorescence-positive cells representing about 0.5% of the infected population were recovered after sorting, cultured for an additional 24 h, and lysed to recover vector virions. Prior to enrichment, the crude viral lysate contained about one  $\beta$ -galactosidase expressing recombinant vector virion per 1,000 parental Ad5 $\Delta$ E2a $\Delta$ E3 virions. Crude viral lysate from the enriched cells contained approximately equal amounts of recombinant vector and Ad5 $\Delta$ E2a $\Delta$ E3, representing a 500-fold increase in vector purity. Vector recov-

TABLE 1. Oligonucleotide primers used for PCR amplification

Primer <sup>a</sup>	Coordinates <sup>b</sup>	Laboratory reference
A	1–18	MG34
B	959–941	E81
C	804–764 <sup>c</sup>	Av9
D	2001–2030 <sup>d</sup>	MG46
E	2430–2401 <sup>d</sup>	MG47
F	22288–22317	MG38
G	25081–25063	MB781
H	23568–23588	MB987
I	27619–27652	E51
J	30552–30529	AV18

<sup>a</sup> The designations correspond to those in Fig. 1.

<sup>b</sup> Sequence coordinates correspond to Ad5 sequences listed under GenBank accession no. M73260 unless otherwise noted. Each sequence is listed from left to right, 5' to 3'.

<sup>c</sup> The sequence corresponds to Rous sarcoma virus long terminal repeat promoter sequences within Av1nBg and pAvS6LacZ (24, 30).

<sup>d</sup> The sequence corresponds to LacZ gene sequences found within Av1nBg and pAvS6LacZ (24, 30).

ered from the enriched cell population was plaque purified twice on AE1-2a cells to isolate Av3nBg.

Amplification of Av3nBg in dexamethasone-induced AE1-2a cells resulted in a typical cytopathic effect characteristic of Av1-infected 293 cells (16) but with delayed onset and growth kinetics as seen for other E2a deletion adenovirus mutants (2). Optimal yields of Av3nBg virions occurred 72 to 96 h post-infection. Under similar conditions, Av1nBg production in 293 cells is optimal about 48 h postinfection. Despite this lag, Av3nBg vector growth was efficient, ultimately yielding approximately 150 to 300 PFU per cell, with purified vector stocks in the range of  $10^{11}$  PFU/ml. This compares favorably with production of Av1nBg in 293 cells, in which yields of 200 to 300 PFU per cell are typical.

The genome of Av3nBg was structurally characterized by Southern analysis (data not shown) and PCR (Fig. 1). PCR amplification confirmed the expected structure for the expression cassette (Fig. 1A). Primers A and B (Table 1) located in the left inverted terminal repeat and Ad5-specific E1a region, respectively, amplified a 941-bp fragment from Ad5, but not Av3nBg or pAvS6nBg, indicating that Av3nBg was free of both Ad5 virus and Ad5E2aΔE3 virus (Fig. 1A, lanes 1 to 3). In agreement with these results, transgene-specific primers D and E (Table 1) amplified a 430-bp DNA fragment from Av3nBg and pAvS6nBg but not Ad5 DNA (lanes 7 to 9). By using primers A to C (Table 1), which are specific for Av3nBg, an 804-bp DNA fragment was amplified from Av3nBg (lane 6) and, as a control, also from pAvS6nBg (lane 5), but not from Ad5 (lane 4).

Evaluation of the E2a and E3 regions of Av3nBg by PCR also confirmed the expected respective deletions (Fig. 1B). Thus, the F and G primers (Table 1) gave a 1,323-bp DNA fragment with Av3nBg and pΔE2aΔE3, confirming the E2a deletion (lanes 11 and 12), and primers I and J (Table 1) gave a 1,055-bp DNA fragment with Av3nBg and pΔE2aΔE3, confirming the E3 deletion (lanes 17 and 18). Use of several sets of primers demonstrated that Av3nBg was purified and free of the potentially contaminating wild-type virus Ad5. Primers H and G (Table 1) did not amplify a 1,513-bp Ad5-specific DNA fragment from either Av3nBg or pΔE2aΔE3 (lanes 14 and 15). As a positive control, Ad5 did yield such a fragment (lane 13). Primers F and G (Table 1) yielded the expected 2,793-bp DNA fragment with Ad5 DNA (lane 10) but no such band from

either Av3nBg or pΔE2aΔE3 DNA (lanes 11 and 12). Finally, primers I and J (Table 1) yielded the expected 2,933-bp DNA fragment from Ad5 (lane 16) but not from either Av3nBg or pΔE2aΔE3 (lanes 17 and 18).

In order to permit comparative molecular characterization of Av3nBg with respect to existing first- and second-generation adenoviral vectors, two such vectors Av1nBg and Av2Lu (30, 31), were utilized. Av1nBg (previously referred to as Av1 LacZ4 [25, 31]) is an Ad5-based, E1 deletion (bp 393 to 3327), E3 deletion (bp 28592 to 30470) vector expressing a nucleus-targeted β-galactosidase reporter and was propagated, purified, titered, and stored at  $-70^{\circ}\text{C}$  as previously described (16). Av2Lu is a second-generation-type vector which harbors the same E1 deletion as Av1nBg and a *ts* mutation in the E2a gene product. This *ts* mutation is the same nucleotide substitution at bp 22966 present in Ad5ts125 (4, 12) and used in construction of another similar second-generation adenovirus vector (3). Av2Lu was constructed from the adenovirus vector construction plasmid pAvS6Lu containing the firefly luciferase gene (25, 30) by homologous recombination with the 35-kb *Clal* fragment of Ad5ts125. Sequence analysis confirmed the presence of the expected single nucleotide substitution present at bp 22966 in Ad5ts125 (data not shown). Av2Lu was propagated at  $32^{\circ}\text{C}$  and then purified, titered, and stored as for Av1nBg (16). All molecular characterization experiments in this study were carried out at  $37^{\circ}\text{C}$  to evaluate the potential utility of the vectors at normal human body temperature. For convenience, throughout the remainder of this article, the abbreviations Av1, Av2, and Av3 will be used to designate the first-, second-, and third-generation adenovirus vectors Av1nBg, Av2Lu, and Av3nBg, respectively.

**Evaluation of the potential for Av1, Av2, and Av3 vector replication.** One important aspect of the safety of adenoviral vectors for in vivo use in human gene therapy is attenuation of autonomous vector DNA replication. In this context, the DNA replication potentials of Av1, Av2, and Av3 vectors in several relevant complementing and noncomplementing cells were compared (Fig. 2). First, as a positive control to demonstrate replication and metabolic labeling of DNA for each vector when both E1 and E2a defects were complemented, dexamethasone-induced AE1-2a cells were infected (10 PFU per cell) with Av1, Av2, or Av3 vectors. Eight hours after infection, cells were washed in phosphate-free Dulbecco modified Eagle medium (DMEM) and labeled for 12 h in phosphate-free DMEM-10 containing  $^{32}\text{P}_i$  (8,500 to 9,120 Ci/mmol, 50  $\mu\text{Ci}/\text{ml}$ ). Metabolically labeled DNA was then extracted, cleaved with *Xba*I, size fractionated by electrophoresis on 0.8% agarose gels, and visualized by autoradiography (Fig. 2A). An *Xba*I restriction cleavage pattern characteristic for each vector was observed after metabolic labeling of vector DNA in infected AE1-2a cells (lanes 2 to 4) but not in uninfected cells (lane 1). The apparent reduction in DNA replication for Av3 in Fig. 2A (lane 4) compared with Av1 (lane 1) and Av2 (lane 2) may be due in part to the lag in growth associated with E2a deletion adenoviral mutants as discussed above (2). Additional experiments evaluating Av1 and Av3 DNA replication in the production cell AE1-2a confirm the differential DNA synthesis in Av1 and Av3 (data not shown). To evaluate the effect of the E2a deletion alone on potential vector DNA replication, vectors were evaluated in 293 cells (7), which complement the E1 defect, under conditions described above (Fig. 2B). The results show approximately equal metabolic labeling of characteristic *Xba*I fragments of Av1 and Av2 vectors (lanes 6 and 7). In contrast, Av3 did not show metabolic labeling of vector DNA in 293 cells (lane 8). As expected, no labeling was observed in uninfected cells (lane 5). The potential for “leaky” replication

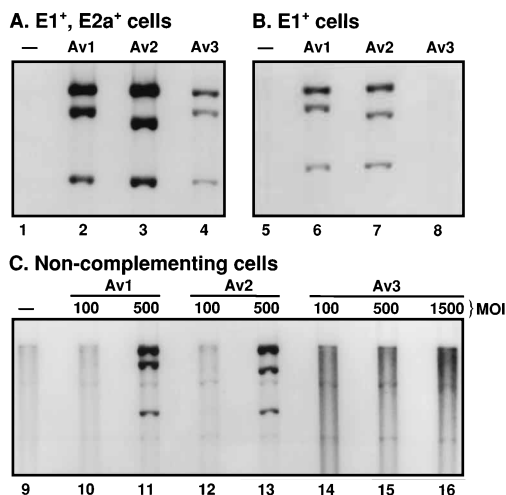


FIG. 2. Evaluation of the potential for de novo vector DNA synthesis of Av1, Av2, and Av3 in various complementing and noncomplementing cells. Metabolic  $^{32}\text{P}$  labeling of vector-infected cells, Hirt extraction, *Xba*I cleavage of vector DNA, and gel electrophoresis and autoradiography were done as described previously (16). Vector infections were carried out at 10 PFU per cell (A and B) or as indicated above each lane (C). Uninfected control cells (lanes 1, 5, and 9) did not show labeled vector DNA. (A) AE1-2a cells induced by dexamethasone to express both Ad5 E1 protein and Ad5 E2a protein to complement E1 and E2a vector deletions, respectively. Note labeling of vector DNA for all three viruses. (B) 293 cells which express E1 proteins to complement only the E1 deletion. Note that metabolic labeling of vector DNA is demonstrated for Av1- and Av2-infected cells but not Av3-infected or uninfected control cells. (C) Naive A549 cells which do not complement either the E1 defect or the E2a defect. See the text for further details.

of vector DNA at a high multiplicity of infection (MOI), previously observed for both E1 deletion adenovirus and E4 deletion adenovirus (10, 22), was evaluated in noncomplementing naive A549 cells infected at an MOI of 100 to 500 (Av1 and Av2) or 100 to 1,500 (Av3) (Fig. 2C). Conditions were similar to those described above for AE1-2a and 293 cells except that metabolic labeling with  $^{32}\text{P}$  was started 24 h after infection and continued for a total of 24 h. As previously demonstrated with HeLa cells (16), Av1 can replicate in A549 cells at a high MOI (lane 11). Similarly, at 37°C, Av2 can also replicate DNA at an MOI of 500 PFU per cell (lane 13). In marked contrast, Av3 did not demonstrate DNA replication even at an MOI of up to 1,500 PFU per cell (lanes 14 to 16).

The potential for vector DNA synthesis was evaluated by measuring the level of vector DNA accumulation in naive A549 cells (Fig. 3). First, as a positive control, the Av1nBg vector was evaluated, because metabolic labeling (Fig. 2) and previous studies (16, 19) suggested that DNA would accumulate because of replication at a high, but not low, MOI. Cells infected with the Av1 vector at an MOI of 100 show measurable vector DNA accumulation at 3 and 6 days after infection (Fig. 3A, lanes 3, 5, and 7). At a low dose of 10 PFU per cell, no such accumulation was observed; rather, vector DNA per cell decreased with time (Fig. 3A, lanes 2, 4, and 6). These observations are consistent with the previously established threshold of 30 PFU per cell required for Av1 vector replication in HeLa cells (16). Importantly, Av3nBg evaluated under similar conditions did not demonstrate vector DNA accumulation at either a low MOI or a high MOI (Fig. 3B).

To further evaluate the potential for replication of the Av3 vector, the yield of infectious virions was measured after infection of noncomplementing cells (Fig. 4). Naive A549 cells inoculated with Av3nBg (MOI, 10 PFU per cell) showed a con-

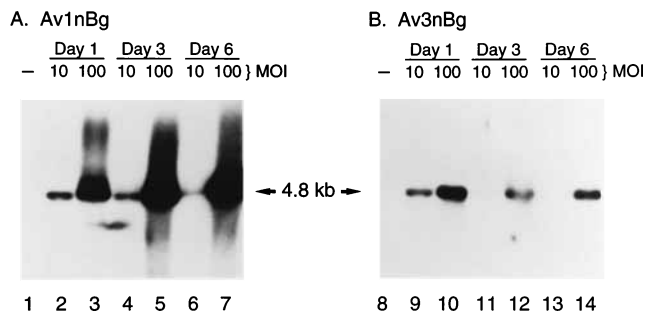


FIG. 3. Comparative evaluation of adenoviral vector DNA accumulation after infection of noncomplementing cells. Cells were infected with Av1nBg or Av3nBg at a low or high MOI (10 or 100, respectively) and cultured for 1 to 6 days. At the times indicated, cells were collected, and total DNA (cellular plus vector) was purified, cleaved with *Bam*HI and *Sal*I, and evaluated by Southern analysis using a  $^{32}\text{P}$ -labeled Ad5 hexon probe, as previously described (10). (A) Av1 vector DNA accumulation in A549 cells. Note that at a low MOI (lanes 2, 4, and 6) Av1nBg vector DNA levels fall with time while at a high MOI (lanes 3, 5, and 7) vector DNA levels increase. As expected, no vector DNA band is seen in uninfected A549 cells (lanes 1 and 8). (B) Lack of Av3 vector accumulation in A549 cells. Note that at both low (lanes 9, 11, and 13) and high (lanes 10, 12, and 14) MOIs Av3nBg vector DNA levels fall with time. Each lane represents the total DNA extracted from cells equivalent to 500  $\mu\text{g}$  of cellular protein.

tinual exponential decrease in the number of recoverable infectious virions for up to 8 days. In marked contrast, as a positive control, naive A549 cells infected with replication-competent Add327 (MOI, 0.1 PFU per cell) showed a continual exponential increase in recoverable infectious virus throughout the evaluation period.

**Evaluation of the potential for hexon gene expression in the Av1, Av2, and Av3 vectors.** Expression of adenovirus major late genes follows and is tightly linked to viral DNA replication (5, 9). To evaluate the hypothesis that combining E1 and E2a deletions would further reduce or eliminate major late gene expression from the vector, hexon protein expression was evaluated in Av1, Av2, and Av3 vector-infected cells by metabolic labeling with  $^{35}\text{S}$ -methionine and hexon immunoprecipitation as previously described (16) (Fig. 5). First, as a positive control, all three vectors were used to infect double-complementing AE1-2a cells (10 PFU per cell) (Fig. 5A). Similar levels of

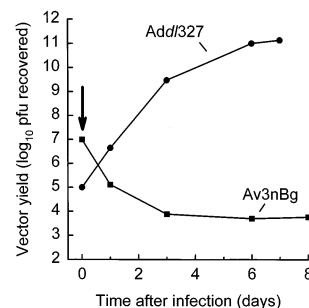


FIG. 4. Evaluation of the potential for production of infectious Av3 vector particles in noncomplementing cells. Naive A549 cells ( $10^6/60\text{-mm}$  culture dish) were inoculated (arrow) with Av3nBg (10 PFU per cell). As a positive control to demonstrate accumulation of infectious virions, naive A549 cells were infected with replication-competent Add327 under similar conditions at a 100-fold-lower MOI. Following a 3-h adsorption, infection medium was removed, the cell monolayer was washed with phosphate-buffered saline, medium was replaced, and the cells were cultured. Cells plus media were harvested at the indicated times postinfection, virus was released from cells by four freeze-thaw cycles, and the crude lysates were titrated on AE1-2a cells induced with dexamethasone. The data are mean titer values from two independent determinations for each sample.

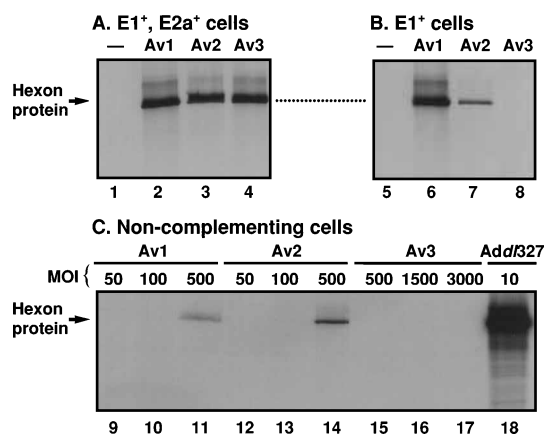


FIG. 5. Evaluation of the potential for adenovirus vector major late (hexon) protein expression by Av1, Av2, and Av3 in various complementing and non-complementing cells. Cells were infected with vectors, metabolically labeled with  $^{35}\text{S}$ -Met, and evaluated for nascent hexon protein synthesis by immunoprecipitation and gel electrophoresis as described previously (16). Labeled hexon bands were not seen in uninfected cells (lanes 1 and 5 in panels A and B). (A) AE1-2a cells induced by dexamethasone to complement E1 and E2a vector deletions. Note that all three vectors express abundant quantities of hexon protein. (B) 293 cells induced to complement only E1, the vector defect. Note that, while Av1 still expresses abundant hexon protein, Av2 expresses less and in Av3 no hexon is detectable. (C) Noncomplementing A549 cells infected at an increasing MOI (given in PFU per cell). Note that no hexon protein expression is detectable in Av3-infected cells even at a high MOI. As a positive control, replication-competent Add327 expresses abundant hexon protein. See the text for further details.

hexon protein expression were shown for each vector, demonstrating comparable levels of complementation for each vector and consistent with the use of equal amounts of each vector for infection. To evaluate the effect of the E2a deletion, independent of the effect of the E1 deletion, 293 cells were used to transcomplement the E1 defect. In separate plates of 293 cells infected (10 PFU per cell) with each vector, both Av1 and Av2 clearly demonstrated hexon gene expression, although expression from Av2 was less (Fig. 5B, lanes 6 and 7). In agreement with the DNA metabolic labeling, no hexon protein was demonstrated from Av3 (lane 8). Results in noncomplementing naive A549 cells (Fig. 3C) were also consistent with DNA metabolic labeling studies. Thus, both Av1 and Av2 expressed hexon protein at a high MOI (lanes 11 and 14). However, Av3 did not show any detectable hexon expression during the  $^{35}\text{S}$ -methionine labeling period from 72 to 96 h postinfection, even at a very high MOI of 3,000 PFU per cell (lanes 15 to 17). As a further control, even small amounts (10 PFU per cell) of Add327 demonstrated very abundant hexon protein synthesis (lane 18).

Hexon expression parallels the vector dose-dependent relationship observed for DNA synthesis by Av1 and Av2 vectors, suggesting that hexon expression is also subject to the cooperativity phenomenon observed for DNA replication with the Av1 (16, 19) and Av2 vectors at a high MOI. Alternatively, the observance of hexon expression from the Av1 and Av2 vectors at a high MOI may be due to a threshold in the sensitivity of the hexon detection assay. Despite this, the Av3 vector did not display hexon expression even at an MOI threefold higher than that for the Av1 and Av2 vectors. The observation that hexon is expressed by the Av1 vector but not by the Av3 vector confirms the previous observation for E2a deletion adenovirus mutants (18) that cellular factors probably regulate major late gene expression in part through the E2a region or its gene product, DBP. This observation suggests the possibility that

expression of other major late genes may also be attenuated by the deletions present in the Av3 vector.

**The Av1 and Av3 vectors have similar gene transfer efficiencies.** To be useful, the Av3 vector should be able to transfer and express a heterologous transgene as efficiently as existing Av1 vectors. Therefore, Av3nBg and Av1nBg were compared for their abilities to transfer and express a  $\beta$ -galactosidase-encoding transgene in naive A549 cells in vitro by a quantitative  $\beta$ -galactosidase assay (14). Prior to gene transfer, both vectors were quantified by optical density at 260 nm to ensure that equal amounts (equivalent to 10 PFU per cell) of each vector were used for infection. Cells were recovered 48 h post-infection by scraping into lysis buffer, and  $\beta$ -galactosidase activity was measured (14) and normalized for the amount of input vector as quantified by optical density. Importantly, gene transfer and expression were similar ( $P > 0.2$ ) for Av1 and Av3 ( $65 \pm 1.5$  and  $49 \pm 1.1$  U of  $\beta$ -galactosidase per  $\mu\text{g}$  of protein  $\times$  optical density at 260 nm, respectively) (Fig. 6).

**Implications for human gene therapy.** Molecular characterization studies confirm that complete elimination of DBP expression significantly improves the biological features of adenovirus vectors related to their potential use in human gene therapy. These combined deletions increase transgene cloning capacity in Av3 to about 8,900 bp (assuming a practical vector genome size of 105% that of the wild-type adenovirus genome). The deletions within the E1 and E2a regions of the Av3 vector minimize the overlap with corresponding E1 and E2a sequences in the production cell AE1-2a, thus reducing the potential for generation of replication-competent adenovirus by homologous recombination, as has been reported to occur for Av1 vectors in 293 cells (13). Importantly, these in vitro studies show that the Av3nBg vector does not express detectable adenovirus hexon capsid protein in noncomplementing cells, while both Av1nBg and Av2Lu vectors do. Similarly, no vector DNA replication was detectable for the Av3nBg vector in noncomplementing cells, while such replication was clearly seen for both Av1nBg and Av2Lu vectors in these cells.

These findings may have important implications for the use of adenoviral vectors for human gene therapy for genetic and acquired disease. First, the Av3 vector should have a relatively reduced risk of spread because the absence of Av3 vector DNA replication should block production of infectious vector virions

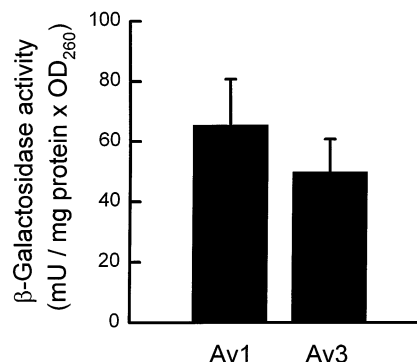


FIG. 6. Comparison of gene transfer and expression by Av1 and Av3 vectors. Av1 (Av1nBg) and Av3 (Av3nBg) were used to infect naive A549 cells at equivalent doses (approximately 10 PFU per cell) based on the optical density of the vector at 260 nm. Transgene transfer and expression were evaluated by measuring  $\beta$ -galactosidase activity in cell lysates and expressed as milliunits of  $\beta$ -galactosidase per milligram of total cellular protein  $\times$  optical density at 260 nm (OD<sub>260</sub>) of the input vector as described in the text. The data represent two separate determinations for three different production lots of each vector type and are presented as means with standard deviations.

in treated cells. The lack of Av3 replication in 293 cells suggests that this will be true even in individuals harboring residual E1 sequences in respiratory epithelium (15), which potentially could complement the replication defect of Av1 vectors. Second, the Av3 vector may have an improved toxicity profile and prolonged transgene expression after *in vivo* vector delivery. For example, absence of expression of adenoviral major late gene products, e.g., hexon, in treated patient cells may reduce the stimulation of the CTL response directed towards the transduced cell, as has been observed for first-generation adenoviral vectors (27, 28). This in turn may reduce CTL-mediated elimination of gene-treated cells, resulting in prolonged expression of the therapeutic gene in the treated tissue. Third, theoretically, Av3 should have a further reduction in cell transformation potential in that the DBP has been implicated in cellular transformation (6). Fourth, elimination of the left inverted terminal repeat sequences in the AE1-2a-complementing cells required to produce Av3 vectors should reduce the potential for recombination resulting in replication-competent virus in vector stocks. Fifth, the Av3-type vector demonstrates gene transfer and expression comparable to that of Av1-type vectors.

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